Effects of Phorbol Myristate Acetate on the Synthesis of 5-Oxo-6,8,11,14-eicosatetraenoic Acid by Human Polymorphonuclear Leukocytes†

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ABSTRACT: 5-Oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) is a newly discovered chemotactic agent for human polymorphonuclear leukocytes (PMNL) which has potent stimulatory effects on cytosolic calcium levels in these cells. Although we have shown that it is synthesized from 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) by a highly specific microsomal dehydrogenase, little is known about the synthesis of this substance by intact PMNL. In the present study we found that in contrast to PMNL microsomes, intact, unstimulated PMNL produced relatively small amounts of 5-oxo-ETE from 5-HETE, but instead converted 5-HETE primarily to its ω-oxidation product, 5,20-diHETE. However, preincubation of PMNL with phorbol myristate acetate (PMA; EC_{50}, ca. 4 nM) dramatically increased the ratio of 5-oxo-ETE to 5,20-diHETE from 0.07 in its absence to 1.85 in the presence of 100 nM PMA. Both effects were completely reversed by staurosporin, indicating that they were mediated by a protein kinase. PMA also stimulated the formation of 5-oxo-ETE, 5-HETE, and leukotriene B_4 (LTB_4) from exogenous arachidonic acid. The greatest enhancement was observed for 5-oxo-ETE, which, under all conditions, was produced in greater quantities than LTB_4. PMA stimulated the formation of 5-oxo-ETE by PMNL stimulated with either A23187 or zymosan. A23187-stimulated PMNL initially produced more LTB_4 than 5-oxo-ETE, but at longer time points, 5-oxo-ETE predominated. These results demonstrate that PMA-activated human PMNL can synthesize substantial amounts of 5-oxo-ETE and raise the possibility that this substance may be an important inflammatory mediator.

Arachidonic acid is converted to two major initial metabolites by human polymorphonuclear leukocytes (PMNL),[1] leukotriene B_4 (LTB_4), and 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) (Borgeat et al., 1976; Borgeat & Samuelsson, 1979). LTB_4 is a potent chemotactic and proaggregatory agent for these cells (Ford-Hutchinson et al., 1980; Palmblad et al., 1981) and appears to act by stimulating the formation of inositol trisphosphate (Mong et al., 1986), resulting in increased cytosolic calcium levels (Naccache et al., 1984). LTB_4 is rapidly converted by LTB_4 20-hydroxylase in human PMNL (Powell, 1984; Shak & Goldstein, 1985; Soberman et al., 1985) to the less biologically active 20-hydroxy-LTB_4 (Ford-Hutchinson et al., 1983), which is further metabolized to ω-carboxy-LTB_4, which has even lower biological activity (Jubiz et al., 1982). Thus the biological activity of LTB_4 is limited in human PMNL by its rapid metabolism to ω-oxidation products.

Unlike LTB_4, 5-HETE has relatively weak biological activities on human PMNL. This compound stimulates cytosolic calcium levels in these cells with an EC_{50} of about 200 nM, compared to an EC_{50} of about 0.2 nM for LTB_4 (O'Flaherty et al., 1988; Powell et al., 1993). 5-HETE also potentiates the effects of platelet-activating factor on superoxide release (Rossi et al., 1991) from PMNL and is a weak chemotactic agent for these cells (Goetzl & Pickett, 1980), but is far less potent than LTB_4. Like LTB_4, 5-HETE is converted to its 20-hydroxy metabolite, 5,20-diHETE, probably by LTB_4 20-hydroxylase (O'Flaherty et al., 1986). However, it is much poorer substrate than LTB_4 and 12-HETE (Soberman et al., 1987) for metabolism by this pathway.

We recently discovered a second pathway for the metabolism of 5-HETE in human PMNL involving a microsomal dehydrogenase which, in the presence of $NADP^+$ converts this compound to its 5-oxo metabolite, 5-oxo-6,8,11,14-eicosatetraenoic acid (5-oxo-ETE) (Powell et al., 1992). 5-Oxo-ETE strongly stimulates cytosolic calcium levels in PMNL with an EC_{50} value of about 2 nM and is about 100 times more potent than 5-HETE in this respect (Powell et al., 1993). It is also about 100 times more potent than 5-HETE as a chemotactic agent for these cells (Powell et al., 1993) and has recently been reported to induce release of enzymes from both primary and secondary granules (O'Flaherty et al., 1993). Although it is not quite as potent as LTB_4 as a PMNL agonist, 5-oxo-ETE stimulates PMNL by a mechanism which is clearly independent of the LTB_4 receptor, since its effects are not subject to desensitization by prior treatment with LTB_4 and are not prevented by the LTB_4 antagonist LY255283 (Powell et al., 1993). Cross desensitization experiments suggest that 5-oxo-ETE interacts with its own receptor, which has affinities for eicosanoids in the order 5-oxo-ETE > 5-oxo-15-hydroxy-ETE > 5-HETE > LTB_4 (Powell et al., 1993). It is likely that the previously reported weak agonist properties of

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[1] The abbreviations used are as follows: PMNL, polymorphonuclear leukocytes; LTB_4, leukotriene B_4; 5-HETE, 5(*S*)-hydroxy-6,8,11,14-eicosatetraenoic acid; 15-HETE, 15(*S*)-hydroxy-5,8,11,13-eicosatetraenoic acid; 13-HODE, 13(*S*)-hydroxy-9,11-octadecadienoic acid; 5,20-diHETE, 5,20-dihydroxy-6,8,11,14-eicosatetraenoic acid; 5-oxo-ETE, 5-oxo-6,8,11,14-eicosatetraenoic acid; 5-oxo-20-hydroxy-ETE, 5-oxo-20-hydroxy-6,8,11,14-eicosatetraenoic acid; PMA, phorbol 12-myristate 13-acetate; RP-HPLC, reversed-phase high-pressure liquid chromatography; t_R, retention time.

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5-HETE on human PMNL (O'Flaherty et al., 1988; Rossi et al., 1991; Goetzl & Pickett, 1980) can be explained by its interaction with such a putative receptor, since its effects on cytosolic calcium levels in PMNL can be completely blocked by prior stimulation of the cells with 5-oxo-ETE (Powell et al., 1993). This is supported by recent findings of O'Flaherty et al. who have also shown that there is cross desensitization in neutrophils between 5-oxo-ETE and 5-HETE, but not between these two agonists and LTB_4 (O'Flaherty et al., 1993).

Our previous data indicate that PMNL have a high *capacity* to synthesize 5-oxo-ETE and that this substance is a potent PMNL agonist. However, if 5-oxo-ETE has a physiological role in inflammation one would expect it to be synthesized by intact cells. It is not clear whether this is the case, since its formation by PMNL microsomes is reversible. Thus in the presence of NADPH, microsomal fractions rapidly convert 5-oxo-ETE to 5(*S*)-HETE. It has been reported that the major metabolite formed after incubation of 5-HETE with unstimulated intact PMNL is 5,20-diHETE (O'Flaherty et al., 1986), and this has been confirmed by preliminary experiments from our laboratory (Powell et al., 1992). However, 5-HETE would normally be produced by activated cells, and it is possible that the activation process could affect its metabolism. In the present study we have examined the effects of the protein kinase C stimulator, phorbol myristate acetate (PMA) on the metabolism of 5-HETE by intact PMNL. We found that this substance strongly stimulates the formation of 5-oxo-ETE from both 5-HETE and arachidonic acid and inhibits the formation of 5,20-diHETE.

MATERIALS AND METHODS

Materials. 5(*S*)-HETE was synthesized by incubation of arachidonic acid (NuChek Prep Inc., Elysian, MN) with porcine PMNL in the presence of 5,8,11,14-eicosatetraynoic acid (Powell, 1984; Borgeat et al., 1981). 13-Hydroxy-9,11-octadecadienoic acid (13-HODE) was synthesized by incubation of linoleic acid with soybean lipoxygenase, reduction of the hydroperoxy product with sodium borohydride (Hamberg & Samuelsson, 1967), and purification by reversed-phase high-pressure liquid chromatography (RP-HPLC). Arachidonic acid used in experiments with human PMNL was purified immediately before use by RP-HPLC using 70% acetonitrile containing 0.02% acetic acid. It had a retention time of 14 min with a flow rate of 1 mL/min. PMA was obtained from the Sigma Chemical Company, St. Louis, MO, whereas staurosporin and A23187 were purchased from Boehringer-Mannheim GmbH and Calbiochem Corp., La Jolla, CA, respectively. Zymosan A (8 mg/mL; Sigma Chemical Company) was sonicated, boiled for 60 min, and centrifuged. The pellet was incubated with fresh human serum for 30 min at 37 °C and washed twice in medium before use.

Preparation of PMNL. Human PMNL were prepared by treatment of blood with Dextran T-500 (Pharmacia), followed by centrifugation over Ficoll-Paque (Pharmacia) and lysis of remaining red blood cells with ammonium chloride (Böyum, 1968). After washing with 0.15 mM NaCl, the cells were resuspended in medium (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4 at a pH of 7.4.

Incubation Conditions. Prior to incubation, calcium and magnesium were added to give final concentrations of 1.8 and 1.0 mM, respectively, and a concentration of PMNL of 5×10^6 cells/mL. Staurosporin and PMA were added 11 and 6 min, respectively, before initiation of eicosanoid synthesis by the addition of 5-HETE, arachidonic acid, or A23187. Incubations were terminated after a further 20 min, unless otherwise indicated, by the addition of methanol (0.6 mL).

Analysis of Metabolites by RP-HPLC. Water (2.4 mL) was added to the incubation mixtures obtained after incubation of PMNL to give a final concentration of methanol of 15%. 13-HODE (200 ng) was added to each sample as an internal standard. We initially analyzed a series of parallel samples to which 13-HODE had not been added to confirm that under the conditions we used, PMNL did not synthesize products which would interfere with the estimation of the peak area for 13-HODE. After centrifugation of the samples, eicosanoids were analyzed by precolumn extraction/RP-HPLC (Powell, 1987) using a Waters-Millipore gradient controller, WISP automatic injector, WAVS automated switching valve, Model 991 diode array detector, and a Model 600 solvent delivery system. The mobile phase was a linear gradient between 100% solvent A [water/acetonitrile/acetic acid (80:20:0.02)] and a mixture of 5% solvent A and 95% solvent B [acetonitrile/methanol/water/acetic acid (38.5:54:7.5:0.02)] over 45 min unless otherwise indicated. The flow rate was 1 mL/min. The stationary phase was a column of Novapak C_{18} (3.9 × 150 mm; Waters-Millipore). Products were quantitated by comparing the areas of their peaks of UV absorbance at their λ_{max} with that of the internal standard, 13-HODE, at 237 nm. The extinction coefficients used were 13-HODE (23 000), 5-HETE (23 000) (Gibian & Vandenberg, 1987), 5-oxo-ETE (19 600),[2] 5,20-diHETE (23 000), 5-oxo-20-hydroxy-ETE (19 600), and LTB_4 and its ω-oxidation products (39 500 at 280 nm) (Borgeat & Samuelsson, 1979). The identities of the products measured were confirmed by examination of their complete UV spectra.

RESULTS

PMA Stimulates the Conversion of 5-HETE to 5-Oxo-ETE by PMNL. 5-HETE (2 μM) was incubated with intact human PMNL (5×10^6 cells/mL) for 20 min in the presence or absence of PMA (30 nM) and the products were analyzed by RP-HPLC (Figure 1). The major metabolite formed in the absence of PMA from 5-HETE was its 20-hydroxylation product, 5,20-dihydroxy-ETE (t_R, 21 min) (Figure 1A). Much smaller amounts of 5-oxo-ETE (t_R, 41 min) and 5-oxo-20-hydroxy-ETE (t_R, 23 min) were formed. Preincubation of PMNL with PMA (30 nM) for 6 min prior to addition of 5-HETE had a dramtic effect on the metabolism of this compound, strongly inhibiting the formation of 5,20-diHETE and stimulating the formation of 5-oxo-ETE, so that the latter compound was the major product under these conditions (Figure 1B).

The time courses for the formation of 5-HETE metabolites by PMNL in the presence and absence of PMA are shown in Figure 2. In the absence of PMA only relatively small amounts of 5-oxo-ETE and its 20-hydroxy metabolite were formed, whereas the major product was 5,20-diHETE (Figure 2A). In contrast, when the cells were preincubated with PMA (30 nM), 5-oxo-ETE was the major product at all time points

[2] The extinction coefficient that we previously reported for 5-oxo-ETE (26 000) (Powell et al., 1992) was based on an extinction coefficient of 30 500 for 5-HETE (Borgeat et al., 1976). In the present paper we have used an extinction coefficient of 23 000 (Gibian & Vandenberg, 1987) for the latter compound and have recalculated the extinction coefficient for 5-oxo-ETE accordingly. The extinction coefficients of the 20-hydroxy metabolites of the above two compounds were assumed to be the same as the parent compounds.

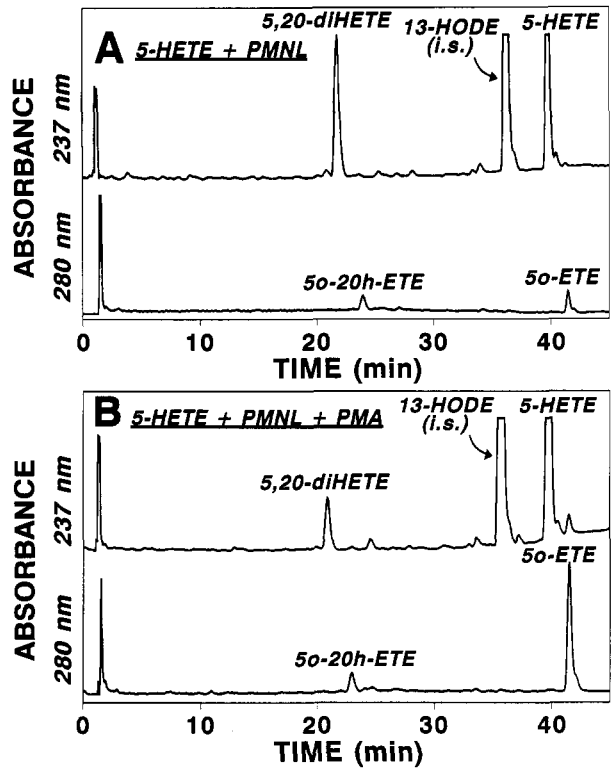

FIGURE 1: High-pressure liquid chromatograms of the products formed after incubation of 5-HETE (2 μM) with human PMNL (1 mL; 5 × 10^6 cells/mL) for 20 min at 37 °C in the absence (A) or presence (B) of PMA (30 nM). The products were analyzed by precolumn extraction/RP-HPLC as described in Materials and Methods. 13-HODE was added as an internal standard (i.s.). Abbreviations are as follows: 5o-ETE, 5-oxo-ETE; 5o-20h-ETE, 5-oxo-20-hydroxy-ETE.

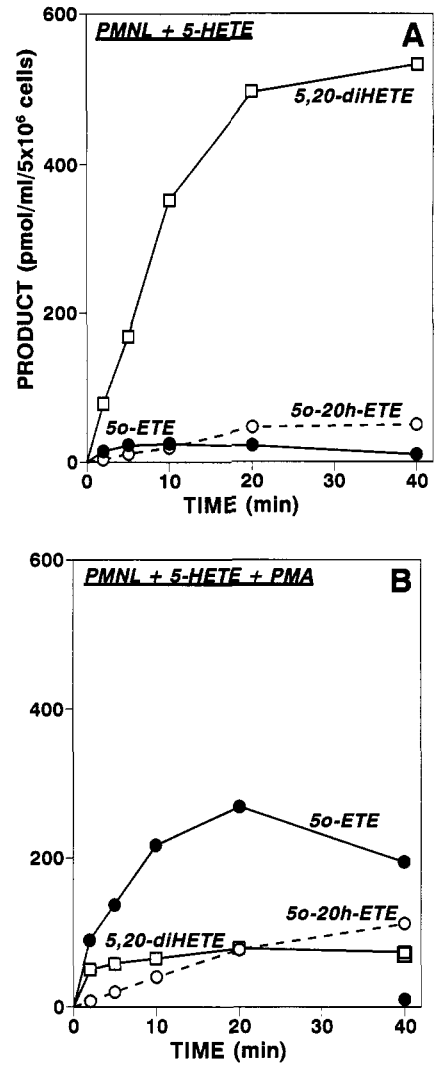

FIGURE 2: Time courses for the formation of metabolites of 5-HETE. PMNL (5 × 10^6 cells/mL) were preincubated with either DMSO (3 μL) (A) or DMSO containing PMA (final concentration, 30 nM) (B) for 6 min at 37 °C. 5-HETE (2 μM) was then added, and the cells were incubated for the times indicated. The products were analyzed by precolumn extraction/RP-HPLC using 13-HODE as an internal standard, as described in Materials and Methods. Symbols are as follows: 5,20-DiHETE (□), 5-oxo-ETE (5o-ETE; ●), 5-oxo-20-hydroxy-ETE (5o-20h-ETE; ○). The data shown are representative of two experiments with similar results.

(Figure 2B). The amounts of the latter product increased with time up to 20 min, and then appeared to decline, probably due to its conversion to 5-oxo-20-hydroxy-ETE, which increased with time up to 40 min. For all of the experiments described below we used incubation times of 20 min unless otherwise indicated.

The Effect of PMA on 5-Oxo-ETE Formation Is Mediated by Protein Kinase. Figure 3A shows that the effects of PMA on the metabolism of 5-HETE are concentration dependent, with an EC_{50} of about 5 nM for stimulation of 5-oxo-ETE formation and about 3 nM for inhibition of 5,20-diHETE production. The ratio of 5-oxo-ETE to 5,20-diHETE rose from about 0.07 in the absence of PMA to 1.85 in the presence of 100 nM PMA. PMA stimulates protein kinase C in neutrophils (White et al., 1984), and if its action on 5-HETE metabolism are mediated by this mechanism, it should be possible to inhibit its effects by adding an inhibitor of this enzyme. Figure 3B shows that staurosporin, which inhibits protein kinase C (Tamaoki et al., 1986), as well as other protein kinases (Herbert et al., 1990), can completely inhibit the effects of PMA in a concentration-dependent manner, with an IC_{50} of about 3 nM. In the presence of PMA (30 nM) and staurosporin (25 nM), the amounts of 5-HETE metabolites formed by PMNL are virtually identical to the amounts formed in the absence of PMA.

PMA Stimulates the Formation of 5-Oxo-ETE from Exogenous Arachidonic Acid. Although 5-oxo-ETE was the major metabolite formed when 5-HETE was added to intact PMNL in the presence of PMA, it was important to determine whether significant amounts of this substance are also synthesized from arachidonic acid. PMNL were incubated with exogenous arachidonic acid (50 μM) in the presence and absence of PMA for 20 min and the products were analyzed by RP-HPLC (Figure 4). In the absence of PMA, the only major metabolites of arachidonic acid were 15-HETE, together with a smaller amount of 5-HETE (Figure 4A). Only very small amounts of 5-oxo-ETE and LTB_4 were detected. PMA (30 nM) strongly stimulated the formation of 5-oxo-ETE and LTB_4, and, to a lesser extent, 5-HETE and 15-HETE (Figure 4B).

The effects of different concentrations of arachidonic acid on the formation of 5-HETE, 5-oxo-ETE, and LTB_4 are shown in Figure 5. In the absence of exogenous arachidonic acid, addition of PMA did not result in the formation of detectable amounts of any of the products measured. In the presence of lower concentrations of arachidonic acid (10 and 20 μM), equivalent amounts of 5-oxo-ETE and 5-HETE were formed, whereas at higher concentrations of arachidonic acid, more 5-HETE was produced. The amounts of 5-oxo-ETE formed were considerably greater than those of LTB_4 at all concentrations of exogenous arachidonic acid tested.

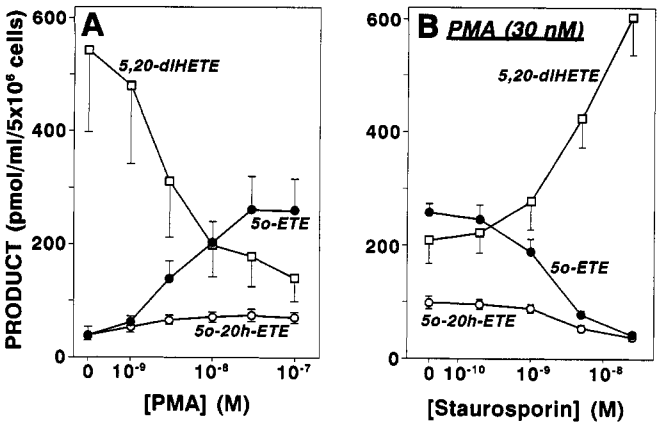

FIGURE 3: Concentration–response curves for the effects of PMA (A) and staurosporin in the presence of PMA (B) on the formation of metabolites of 5-HETE by PMNL. In A PMNL (5×10^6 cells/mL) were preincubated at 37 °C for 6 min with either DMSO (3 μL) or DMSO containing various concentrations of PMA. The cells were then incubated with 5-HETE (2 μM) for 20 min. In B cells were first preincubated at 37 °C with either DMSO (3 μL) or DMSO containing various concentrations of staurosporin. After 5 min, PMA (final concentration, 30 nM) was added, and the cells were preincubated for a further 6 min, prior to incubation with 5-HETE (2 μM) for 20 min. The products were analyzed by precolumn extraction/RP-HPLC as described in Materials and Methods. The results are means ± SEM of experiments with PMNL from either 5 (A) or 4 (B) different donors. All experiments were performed in duplicate. Symbols are as follows: 5,20-DiHETE (□), 5-oxo-ETE (5o-ETE; ●), 5-oxo-20-hydroxy-ETE (5o-20h-ETE; ○). The amounts of all three products were significantly different from controls [without PMA (A) or without staurosporin (B)] at concentrations of PMA between 3 and 100 nM (A) and at concentrations of staurosporin between 5 and 25 nM (B).

The amounts of 5-HETE, 5-oxo-ETE, and LTB_4 formed from arachidonic acid (50 μM) in the presence of different concentrations of PMA are shown in Figure 6. PMA stimulated the formation of both 5-oxo-ETE (EC_{50}, 1.5 nM) and LTB_4 (EC_{50}, 1 nM). The formation of 5-HETE, the most abundant 5-lipoxygenase product at all concentrations of PMA, also appeared to be stimulated, but this was not significant. The ratio of 5-oxo-ETE to 5-HETE increased from 0.32 ± 0.02 in the absence of PMA to 0.74 ± 0.13 in the presence of 100 nM PMA ($p < 0.05$).

PMA Enhances the Formation of 5-Oxo-ETE from Endogenous Arachidonic Acid. In addition to being formed from exogenous 5-HETE and exogenous arachidonic acid, 5-oxo-ETE was also formed from endogenous substrate released in response to A23187. The time courses for the production of 5-HETE, 5-oxo-ETE, and LTB_4 by PMNL stimulated with A23187 (5 μM) are shown in Figure 7. In the absence of PMA, the amounts of 5-HETE and LTB_4 in the incubation mixtures reached maximal levels between 2 and 5 min and then declined (Figure 7A). In contrast, 5-oxo-ETE appeared to be formed more slowly, but its concentration did not change very much after 10 min, suggesting that it is metabolized more slowly than the former two compounds. PMA increased the amounts of both 5-HETE and 5-oxo-ETE in the incubation mixtures by approximately 2-fold and caused a more modest increase in the levels of LTB_4 (Figure 7B).

Incubation of PMNL with serum-treated zymosan (1 mg/mL) for 60 min resulted in the formation of only small amounts of eicosanoids in the absence of PMA. However, in the presence of PMA (30 nM), the response to serum-treated zymosan was considerably enhanced (Figure 8). Comparable amounts of 5-oxo-ETE and LTB_4 were synthesized by

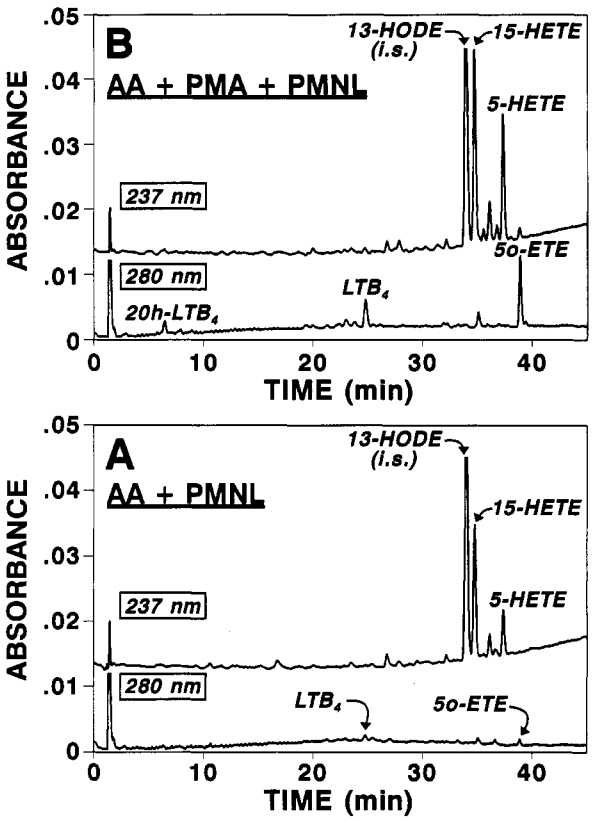

FIGURE 4: High-pressure liquid chromatogram of the products formed after incubation of arachidonic acid (50 μM) with human PMNL (1 mL; 5×10^6 cells/mL) for 20 min at 37 °C in the absence (A) or presence (B) of PMA (30 nM). The products were analyzed by precolumn extraction/RP-HPLC as described in Materials and Methods. 13-HODE was added as an internal standard (i.s.). In each panel, the bottom tracing shows absorbance at 280 nm, whereas the top one shows absorbance at 237 nm.

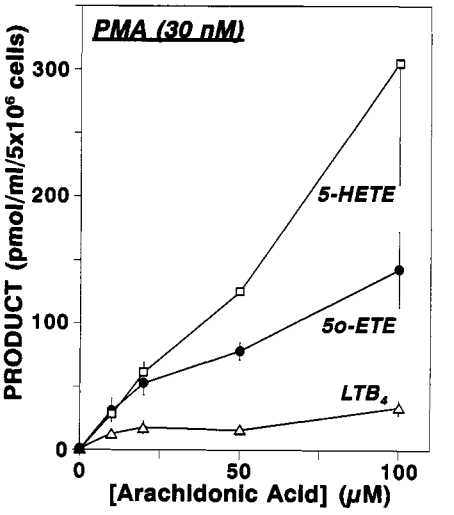

FIGURE 5: Effects of arachidonic acid concentration on the formation of 5-HETE (□), 5-oxo-ETE (●), and LTB_4 (△) by PMNL treated with PMA. PMNL (5×10^6 cells/mL) were preincubated for 6 min with PMA (30 nM) and then incubated for a further 20 min in the presence or absence of various concentrations of arachidonic acid. The products were quantitated by precolumn extraction/RP-HPLC as described in Materials and Methods. The values are means ± SEM of experiments performed in duplicate on PMNL from three different donors.

zymosan-stimulated PMNL, both in the presence and absence of PMA. Incubation of PMNL with PMA alone did not result in the formation of detectable amounts of products.

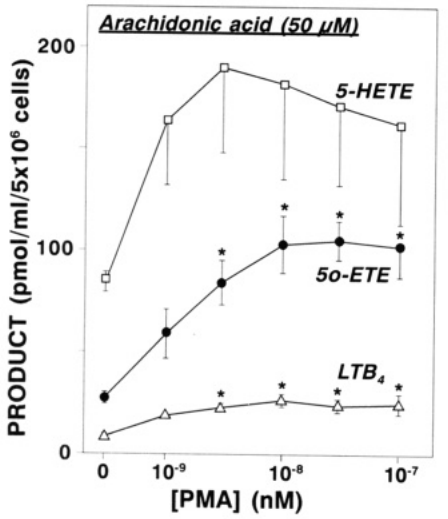

FIGURE 6: Effects of different concentrations of PMA on the formation of 5-HETE (□), 5-oxo-ETE (●), and LTB_4 (△) by PMNL incubated with arachidonic acid (50 μM) for 20 min. PMNL (5×10^6 cells/mL) were preincubated with PMA for 6 min prior to the addition of arachidonic acid. The products were quantitated by precolumn extraction/RP-HPLC as described in Materials and Methods. The values are means ± SEM of experiments performed in duplicate on PMNL from three different donors. An asterisk (*) indicates that the value is significantly different from controls incubated in the absence of PMA ($p < 0.05$).

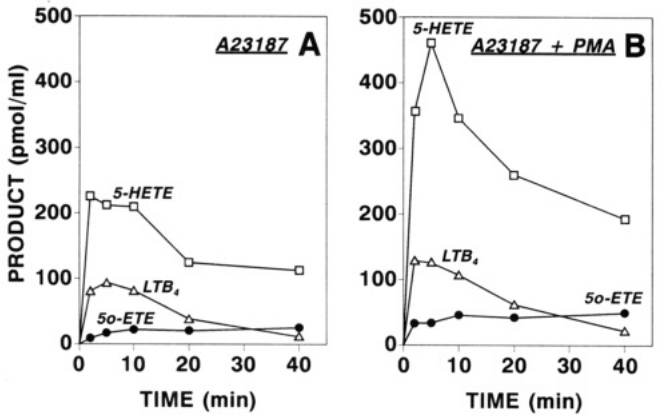

FIGURE 7: Time courses for the formation of 5-HETE (□), 5-oxo-ETE (●), and LTB_4 (△) by PMNL incubated with A23187 in the absence (A) or presence (B) of PMA. PMNL were preincubated with either DMSO (3 μL) (A) or PMA (30 nM) (B) for 6 min and then incubated in the presence of A23187 (5 μM) for various times at 37 °C. The products were quantitated by precolumn extraction/RP-HPLC as described in Materials and Methods. The data shown are representative of experiments with similar results using cells from three different donors.

DISCUSSION

Previous studies in our laboratory have shown that 5-oxo-ETE is a potent stimulator of intracellular calcium levels and chemotaxis in human neutrophils (Powell et al., 1993). Microsomal fractions from PMNL contain high levels of 5-hydroxyeicosanoid dehydrogenase activity (Powell et al., 1992) and these cells thus have a large capacity to generate 5-oxo-ETE. However, the metabolism of 5-HETE in intact PMNL is more complicated due to the formation of ω-oxidation products, resulting in the formation of 5,20-diHETE and 5-oxo-20-hydroxy-ETE, in addition to 5-oxo-ETE (Figure 9). In spite of their high dehydrogenase activity, unstimulated PMNL convert 5-HETE to only modest amounts of 5-oxo-ETE, along with much greater amounts of 5,20-diHETE. This could be due to several factors. In the case of incubations

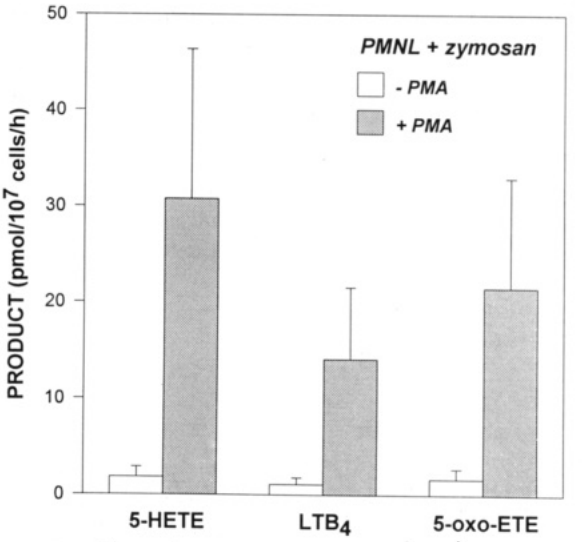

FIGURE 8: Effects of serum-treated zymosan and PMA on the formation of 5-HETE, LTB_4, and 5-oxo-ETE by PMNL. PMNL (5×10^6 cells/mL) were incubated with serum-treated zymosan (1 mg/mL) for 60 min at 37 °C in the absence (open bars) or presence (hatched bars) of PMA (30 nM). The products were quantitated by precolumn extraction/RP-HPLC as described in Materials and Methods. The data are means ± SEM of four experiments, each conducted in duplicate.

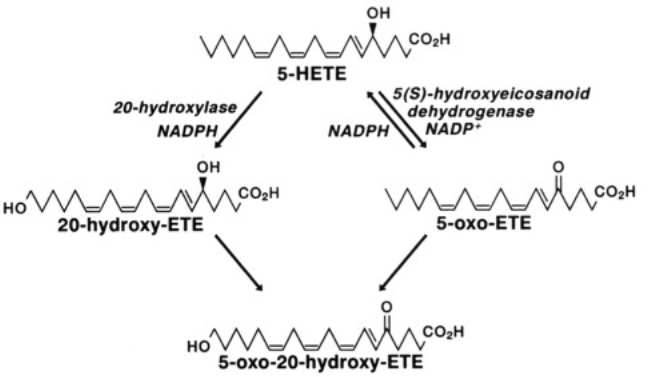

FIGURE 9: Metabolism of 5-HETE by intact PMNL.

with microsomal fractions, the reaction was driven in the direction of oxidation (i.e. 5-HETE → 5-oxo-ETE) by the addition of a high concentration (1 mM) of $NADP^+$. However, we have shown that PMNL microsomes also catalyze the reverse reaction (i.e. 5-oxo-ETE → 5-HETE) in the presence of NADPH. It is not clear whether the latter reaction is due to 5-hydroxyeicosanoid dehydrogenase acting in the reverse direction, or whether a distinct 5-ketoreductase enzyme exists in PMNL. Since intact cells contain both NADPH and $NADP^+$, both the forward and reverse reactions would be expected to take place. In addition, the 20-hydroxylase responsible for the formation of 5,20-diHETE would be active in intact cells in the presence of NADPH, but would not metabolize 5-HETE in microsomal fractions containing only $NADP^+$.

Preincubation of PMNL with PMA resulted in a dramatic shift in the metabolism of 5-HETE, increasing the ratio of 5-oxo-ETE to 5,20-diHETE from 0.07 to 1.85. This is unlikely to be due to nonspecific oxidation by oxidants such as hypochlorous acid since methionine, a scavenger of this substance, and azide, an inhibitor of myeloperoxidase, do not prevent the effects of PMA on the metabolism of 5-HETE.[3] Although the precise mechanism for the stimulatory effect of PMA on the formation of 5-oxo-ETE is unclear, it is presumably mediated by protein kinase C-induced protein

phosphorylation, since its effect is completely blocked by staurosporin. It is possible that PMA could induce the phosphorylation of 5(*S*)-hydroxyeicosanoid dehydrogenase, which is responsible for the formation of 5-oxo-ETE, resulting in an overall increase in its activity. Alternatively, the enzyme properties could be changed by phosphorylation, so that dehydrogenase activity is favored over reductase activity (if indeed the same enzyme catalyzes both activities). It is also possible that the activity of the 20-hydroxylase could be reduced due to phosphorylation, resulting in a diversion in the metabolism of 5-HETE from 5,20-diHETE to 5-oxo-ETE.

Another possible explanation for the effects of PMA on the metabolism of 5-HETE is that these effects are not mediated by changes in the metabolic enzymes themselves, but rather by changes in the intracellular levels of the cofactors which are required for the expression of their activities. Such changes could be mediated by the dramatic stimulatory effect of PMA on NADPH oxidase, an enzyme which is latent in unstimulated PMNL, but becomes highly active in the presence of agents which stimulate protein kinase C, resulting in the respiratory burst and superoxide production (Nauseef et al., 1991). This is due to phosphorylation of one of the cytosolic components of the oxidase (p47-*phox*) and the translocation of p47-*phox* and p67-*phox* from the cytosol to the plasma membrane, resulting in activation of the latent membrane-bound components of the oxidase (Nauseef et al., 1991). Activation of NADPH oxidase would result in the rapid oxidation of NADPH to $NADP^+$, which would be expected to enhance the rate of formation of 5-oxo-ETE from 5-HETE and to reduce the rate of both the reverse reaction and the conversion of 5-HETE to 5,20-diHETE.

5-Oxo-ETE is a major metabolite of exogenous arachidonic acid in PMA-activated PMNL and is produced in substantially greater amounts than LTB_4 under these conditions. Only relatively small amounts of 5,20-diHETE and 5-oxo-20-hydroxy-ETE were formed from exogenous arachidonic acid, possibly due to competition from LTB_4 for the 20-hydroxylase required for their formation. The mechanism of action of PMA may be more complex when exogenous arachidonic acid is the substrate, since PMA stimulates the formation not only of 5-oxo-ETE, but also of its precursor, 5-HETE, as well as LTB_4. Although the increased availability of 5-HETE would presumably contribute to the stimulatory effect of PMA on 5-oxo-ETE formation from exogenous arachidonic acid, it cannot explain it entirely, since there is a concentration-dependent increase in the ratio of 5-oxo-ETE to 5-HETE. Thus the effect of PMA on the formation of 5-oxo-ETE from arachidonic acid is presumably due to a combination of its stimulatory effects on both the formation of 5-HETE and the conversion of the latter to 5-oxo-ETE.

5-Oxo-ETE was also synthesized from endogenous arachidonic acid by PMNL stimulated by A23187, and its formation was stimulated about 2-fold by PMA (30 nM) (Figure 7). The stimulatory effect of PMA under these conditions was not as dramatic as when exogenous 5-HETE (ca. 7-fold stimulation) or exogenous arachidonic acid (ca. 4-fold stimulation) was added as substrate, in spite of the fact that A23187 stimulated the formation of high levels of 5-HETE. The amounts of LTB_4 were initially greater than those of 5-oxo-ETE, presumably due to the strong stimulatory effect of A23187 on cytosolic calcium levels, resulting in translocation of 5-lipoxygenase to the membrane and efficient production of LTB_4 (Dixon et al., 1990; Hill et al., 1992). However, at longer time points, more 5-oxo-ETE than LTB_4 was present, both in the presence and absence of PMA, probably because it was not metabilized as rapidly as LTB_4. The effects of PMA on the formation of eicosanoids in response to A23187 are presumably more complicated than its effects on the metabolism of exogenous substrates because it could also have other effects, such as stimulation of phospholipase A_2 (Lin et al., 1992). However, the reason for the reduced ratio of 5-oxo-ETE to 5-HETE in cells stimulated with A23187 and PMA compared to cells stimulated with arachidonic acid and PMA is not yet clear. PMA also enhances the formation of 5-oxo-ETE by PMNL stimulated with serum-treated zymosan. As was the case for the A23187, the effect of PMA did not appear to be selective, since the amounts of 5-HETE and LTB_4 formed were also increased.

Although the physiological significance of 5-oxo-ETE has not yet been established, several lines of evidence would be consistent with a role for this substance as an inflammatory mediator. First, it is synthesized by a highly specific dehydrogenase, which requires a substrate with a 5-hydroxyl group in the *S* configuration followed by a 6-*trans* double bond (Powell et al., 1992). Second, as shown by the present study, it is synthesized in substantial amounts by intact neutrophils stimulated with the protein kinase C activator, PMA. Third, it is a potent stimulator of cytosolic calcium levels in neutrophils and acts by a mechanism which is clearly independent of the LTB_4 receptor (Powell et al., 1993; O'Flaherty et al., 1993). It is also a chemotactic agent for these cells (Powell et al., 1993) and induces degranulation (O'Flaherty et al., 1993). Finally, preliminary experiments in our laboratory have shown that 5-oxo-ETE is a potent chemotactic agent for eosinophils.[4] The concentrations of 5-oxo-ETE synthesized by activated PMNL in the present study (ca. 25–300 nM, depending on the stimulus) are well above those required for its biological effects [EC_{50} for stimulation of cytosolic calcium levels, 2 nM (Powell et al., 1993)]. Although it is somewhat less potent as a neutrophil agonist than LTB_4, it is capable of stimulating cells that have become desensitized to this leukotriene. Moreover, the half-life of 5-oxo-ETE in suspensions of PMNL appears to be longer than that of LTB_4, presumably because the former compound is not as good a substrate for LTB_4 20-hydroxylase. Thus its biological activity could be more prolonged than that of LTB_4. In the case of eosinophils, 5-oxo-ETE appears to be considerably more potent as a chemotactic agent than LTB_4.[4] This is consistent with a report that another 5-oxo-eicosanoid, 5-oxo-15-hydroxy-ETE, is more potent than LTB_4 as an eosinophil chemotaxin (Schwenk et al., 1992). We have shown that the latter substance stimulates neutrophils by a mechanism similar to that of 5-oxo-ETE, except that the 15-hydroxy compound is somewhat less potent (Powell et al., 1993).

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